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(54) Title: MTS GENE, MUTATIONS THEREIN, AND METHODS FOR DIAGNOSING CANCER USING MTS GENE SEQUENCE

(57) Abstract

The present invention relates to somatic mutations in the Multiple Tumor Suppressor (MTS) gene in human cancers and their use in the diagnosis and prognosis of human cancer. The invention further relates to germ line mutations in the MTS gene and their use in the diagnosis of predisposition to melanoma, leukemia, astrocytoma, glioblastoma, lymphoma, glioma, Hodgkin's lymphoma, CLL, and cancers of the pancreas, breast, thyroid, ovary, uterus, testis, kidney, stomach and rectum. The invention also relates to the therapy of human cancers which have a mutation in the MTS gene, including gene therapy, protein replacement therapy and protein mimetics. Finally, the invention relates to the screening of drugs for cancer therapy.

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SDS and one 10 min wash in 1x SSC, 0.1% Triton[®]X-100. The blots are rinsed for 10 min. at room temp. with 1X SSC.

Blots are incubated for 10 min at room temperature with shaking in the substrate buffer consisting of 0.1 M diethanolamine, 1 mM MgCl₂, 0.02% sodium azide, pH 10.0. Individual blots
5 are placed in heat sealable bags with substrate buffer and 0.2 mM AMPPD (3-(2'-spiroadamantane)-4-methoxy-4-(3'-phosphoryloxy)phenyl-1,2-dioxetane, disodium salt, Bio-Rad).

After a 20 min. incubation at room temperature with shaking, the excess AMPPD solution is removed. The blot is exposed to X-ray film overnight. Positive bands indicate the presence of MTS.

10

EXAMPLE 16

Generation of Polyclonal Antibody against MTS

Segments of MTS coding sequence were expressed as fusion protein in *E. coli*. The
15 overexpressed protein was purified by gel elution and used to immunize rabbits and mice using a procedure similar to the one described by Harlow and Lane, 1988. This procedure has been shown to generate Abs against various other proteins (for example, see Kraemer, et al., 1993).

Briefly, a stretch of MTS coding sequence was cloned as a fusion protein in plasmid PET5A (Novagen, Inc., Madison, WI). The MTS incorporated sequence includes the amino acids
20 corresponding to 448-498 of SEQ ID NO:4. After induction with IPTG, the overexpression of a fusion protein with the expected molecular weight was verified by SDS/PAGE. Fusion protein was purified from the gel by electroelution. The identification of the protein as the MTS fusion product was verified by protein sequencing at the N-terminus. Next, the purified protein was used as immunogen in rabbits. Rabbits were immunized with 100 µg of the protein in complete
25 Freund's adjuvant and boosted twice in 3 week intervals, first with 100 µg of immunogen in incomplete Freund's adjuvant followed by 100 µg of immunogen in PBS. Antibody containing serum is collected two weeks thereafter.

This procedure is repeated to generate antibodies against the mutant forms of the MTS gene. These antibodies, in conjunction with antibodies to wild type MTS, are used to detect the
30 presence and the relative level of the mutant forms in various tissues and biological fluids.

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EXAMPLE 17

Generation of Monoclonal Antibodies Specific for MTS

Monoclonal antibodies are generated according to the following protocol. Mice are
5 immunized with immunogen comprising intact MTS or MTS peptides (wild type or mutant) conjugated to keyhole limpet hemocyanin using glutaraldehyde or EDC as is well known.

The immunogen is mixed with an adjuvant. Each mouse receives four injections of 10 to 100 µg of immunogen and after the fourth injection blood samples are taken from the mice to determine if the serum contains antibody to the immunogen. Serum titer is determined by ELISA
10 or RIA. Mice with sera indicating the presence of antibody to the immunogen are selected for hybridoma production.

Spleens are removed from immune mice and a single cell suspension is prepared (see Harlow and Lane, 1988). Cell fusions are performed essentially as described by Kohler and Milstein, 1975. Briefly, P3.65.3 myeloma cells (American Type Culture Collection, Rockville,
15 MD) are fused with immune spleen cells using polyethylene glycol as described by Harlow and Lane, 1988. Cells are plated at a density of 2×10^5 cells/well in 96 well tissue culture plates. Individual wells are examined for growth and the supernatants of wells with growth are tested for the presence of MTS specific antibodies by ELISA or RIA using wild type or mutant MTS target protein. Cells in positive wells are expanded and subcloned to establish and confirm
20 monoclonality.

Clones with the desired specificities are expanded and grown as ascites in mice or in a hollow fiber system to produce sufficient quantities of antibody for characterization and assay development.

25

EXAMPLE 18

Sandwich Assay for MTS

Monoclonal antibody is attached to a solid surface such as a plate, tube, bead, or particle. Preferably, the antibody is attached to the well surface of a 96-well ELISA plate. 100 µl sample
30 (e.g., serum, urine, tissue cytosol) containing the MTS peptide/protein (wild-type or mutants) is added to the solid phase antibody. The sample is incubated for 2 hrs at room temperature. Next

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the sample fluid is decanted, and the solid phase is washed with buffer to remove unbound material. 100 μ l of a second monoclonal antibody (to a different determinant on the MTS peptide/protein) is added to the solid phase. This antibody is labeled with a detector molecule (e.g., 5 125-I, enzyme, fluorophore, or a chromophore) and the solid phase with the second antibody is incubated for two hrs at room temperature. The second antibody is decanted and the solid phase is washed with buffer to remove unbound material.

The amount of bound label, which is proportional to the amount of MTS peptide/protein present in the sample, is quantitated. Separate assays are performed using monoclonal antibodies which are specific for the wild-type MTS as well as monoclonal antibodies specific for each of the 10 mutations identified in MTS.

It will be appreciated that the methods and compositions of the instant invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent to the artisan that other embodiments exist and do not depart from the spirit of the 15 invention. Thus, the described embodiments are illustrative and should not be construed as restrictive.

includes recombinant or cloned DNA isolates and chemically synthesized analogs or analogs biologically synthesized by heterologous systems.

"MTS Allele" refers to normal alleles of the MTS locus as well as alleles carrying variations that predispose individuals to develop cancer of many sites including, for example, melanoma,
5 ocular melanoma, leukemia, astrocytoma, glioblastoma, lymphoma, glioma, Hodgkin's lymphoma, multiple myeloma, sarcoma, myosarcoma, cholangiocarcinoma, squamous cell carcinoma, CLL, and cancers of the pancreas, breast, brain, prostate, bladder, thyroid, ovary, uterus, testis, kidney, stomach, colon and rectum. Such predisposing alleles are also called "**MTS susceptibility alleles**".

10 "MTS Locus," "MTS gene," "MTS Nucleic Acids" or "MTS Polynucleotide" refer to polynucleotides, all of which are in the MTS region, that are likely to be expressed in normal tissue, certain alleles of which predispose an individual to develop melanoma and other cancers, such as ocular melanoma, leukemia, astrocytoma, glioblastoma, lymphoma, glioma, Hodgkin's lymphoma, multiple myeloma, sarcoma, myosarcoma, cholangiocarcinoma, squamous cell
15 carcinoma, CLL, and cancers of the pancreas, breast, brain, prostate, bladder, thyroid, ovary, uterus, testis, kidney, stomach, colon and rectum. The MTS locus is used interchangeably herein with the prior art designation MLM locus, and the use of "MTS" is intended to include "MLM" as used with reference to locus, gene, region, and the like. Mutations at the MTS locus may be involved in the initiation and/or progression of other types of tumors. The locus is indicated in part
20 by mutations that predispose individuals to develop cancer. These mutations fall within the MTS region described *infra*. The MTS locus is intended to include coding sequences, intervening sequences and regulatory elements controlling transcription and/or translation. The MTS locus is intended to include all allelic variations of the DNA sequence.

These terms, when applied to a nucleic acid, refer to a nucleic acid which encodes a MTS
25 polypeptide (including p16), fragment, homolog or variant, including, e.g., protein fusions or deletions. The nucleic acids of the present invention will possess a sequence which is either derived from, or substantially similar to a natural MTS-encoding gene or one having substantial homology with a natural MTS-encoding gene or a portion thereof. The coding sequence for an MTS polypeptide (MTS1) is shown in SEQ ID NO:1, and the amino acid sequence of an MTS
30 polypeptide (MTS1) is shown in SEQ ID NO:2. The coding sequence for a second MTS polypeptide (MTS1E1 β) is shown in SEQ ID NO:13, and the corresponding amino acid sequence

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is shown in SEQ ID NO:14. The coding sequence for a third MTS polypeptide (MTS2) is shown in SEQ ID NO:15, and the corresponding amino acid sequence is shown in SEQ ID NO:16. The term P16 is used interchangeably with MTS1 and MTS1E1 β and is used to mean both MTS1 which encodes a p16 and MTS1E1 β which encodes a p10. MTS1 and MTS1E1 β are two forms of 5 one gene, the tow forms being dependent upon which of two promoters is used for transcription. MTS2 is a separate portion of the MTS region and it encodes a p15.

The polynucleotide compositions of this invention include RNA, cDNA, genomic DNA, synthetic forms, and mixed polymers, both sense and antisense strands, and may be chemically or biochemically modified or may contain non-natural or derivatized nucleotide bases, as will be 10 readily appreciated by those skilled in the art. Such modifications include, for example, labels, methylation, substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.), charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), pendent moieties (e.g., polypeptides), intercalators (e.g., acridine, 15 psoralen, etc.), chelators, alkylators, and modified linkages (e.g., alpha anomeric nucleic acids, etc.). Also included are synthetic molecules that mimic polynucleotides in their ability to bind to a designated sequence via hydrogen bonding and other chemical interactions. Such molecules are known in the art and include, for example, those in which peptide linkages substitute for phosphate linkages in the backbone of the molecule.

20 The present invention provides recombinant nucleic acids comprising all or part of the MTS region. The recombinant construct may be capable of replicating autonomously in a host cell. Alternatively, the recombinant construct may become integrated into the chromosomal DNA of the host cell. Such a recombinant polynucleotide comprises a polynucleotide of genomic, cDNA, semi-synthetic, or synthetic origin which, by virtue of its origin or manipulation, 1) is not associated with all or a portion of a polynucleotide with which it is associated in nature; 2) is linked to a 25 polynucleotide other than that to which it is linked in nature; or 3) does not occur in nature.

Therefore, recombinant nucleic acids comprising sequences otherwise not naturally occurring are provided by this invention. Although the wild-type sequence may be employed, it will often be altered, e.g., by deletion, substitution or insertion.

30 cDNA or genomic libraries of various types may be screened as natural sources of the nucleic acids of the present invention, or such nucleic acids may be provided by amplification of

candidate probes contemplated within the scope of the invention include probes that include the allele-specific mutations identified in Tables 3 and 5.

Methods of Use: Peptide Diagnosis and Diagnostic Kits

5 The neoplastic condition of lesions can also be detected on the basis of the alteration of wild-type MTS polypeptide. Such alterations can be determined by sequence analysis in accordance with conventional techniques. More preferably, antibodies (polyclonal or monoclonal) are used to detect differences in, or the absence of MTS peptides. In a preferred embodiment of the invention, antibodies will immunoprecipitate MTS proteins from solution as well as react with MTS protein
10 on Western or immunoblots of polyacrylamide gels. In another preferred embodiment, antibodies will detect MTS proteins in paraffin or frozen tissue sections, using immunocytochemical techniques. Techniques for raising and purifying antibodies are well known in the art, and any such techniques may be chosen to achieve the preparation of the invention.

Preferred embodiments relating to methods for detecting MTS or its mutations include
15 enzyme linked immunosorbent assays (ELISA), radioimmunoassays (RIA), immunoradiometric assays (IRMA) and immunoenzymatic assays (IEMA), including sandwich assays using monoclonal and/or polyclonal antibodies. Exemplary sandwich assays are described by David et al., in US Patent Nos. 4,376,110 and 4,486,530, hereby incorporated by reference, and exemplified in Example 18.

20

Methods of Use: Drug Screening

The present invention is particularly useful for screening compounds by using the Cdk polypeptides or binding fragments thereof in any of a variety of drug screening techniques. Preferably, Cdk4 is utilized. The Cdk polypeptide or fragment employed in such a test may either
25 be free in solution, affixed to a solid support, or borne on a cell surface. One method of drug screening utilizes eukaryotic or prokaryotic host cells which are stably transformed with recombinant polynucleotides expressing the polypeptide or fragment, preferably in competitive binding assays. Such cells, either in viable or fixed form, can be used for standard binding assays. One may measure, for example, for the formation of complexes between a Cdk polypeptide or
30 fragment and the agent being tested, or examine the degree to which the formation of a complex

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12). There was no indication of non-overlapping deletions in this panel of cell lines other than those within cosmid c5. Therefore, there is no basis to invoke a more complex scheme involving, for example, a second tumor suppressor locus in 9p21 distant from c5.1 and c5.3.

The observation that homozygous deletions of 9p21 occur in multiple tumor types suggests
5 that the tumor suppressor gene(s) located there may be expressed in a wide variety of tissues. Thus, the tumor suppressor gene(s) may be similar to the p53 gene in that it may participate in the development of multiple types of cancer (see further data below). Other types of cancer have been reported in melanoma-prone families (Nancarrow et al., 1993; Bergman et al., 1990). A thorough deletion analysis of a wide variety of tumor types using c5.1 and c5.3 (shown below) clarifies the
10 importance of this tumor suppressor gene in tumors other than melanoma.

Some of the homozygous deletions observed remove many genetic markers. Fountain et al.
reported that homozygous deletions of chromosome 9p21 in two different melanoma lines
extended 2-3 Mb (Bergman et al., 1990). In this study, at least one line, SK-MEL-5, contained
deletions extending from the most distal marker tested, IFNA-1, past D9S126, a region apparently
15 too large to be contained on a single YAC. The preponderance of large deletions suggests that the
region surrounding MTS is devoid of genes that are essential to cellular viability.

EXAMPLE 6

Isolation of MTS Candidate Genes

20

In the previous Examples, the results of a YAC and P1 chromosomal walk in the
neighborhood of MTS were described. Fine structure-mapping experiments with STSs derived
from c5 sequences showed the presence of small, non-overlapping deletions of c5 sequences in five
different melanoma cell lines. Based on this result, it was probable that a tumor suppressor gene,
25 possibly MTS, lay at least partly within c5.

A further indication that c5 contained at least one gene came from analysis of (CpG)
dinucleotide frequencies in c5 and neighboring cosmids. In mammals, virtually all housekeeping
genes and nearly half of all tissue-specific genes are associated with regions unusually rich in
(CpG) dinucleotides (Bird, 1989; Larsen et al., 1992). Thus, the presence of such "CpG islands" is
30 indicative of genes. Cosmids c5, c12, c57, and c59 were digested with the restriction
endonucleases EagI, BssHI, and SacII, enzymes whose recognition sequences include two (CpG)

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pairs. Only cosmids c5 and c12 contained sites for these enzymes. Cosmid c5 contained one EagI site, at least 10 BssHII sites, and at least 12 SacII sites. The presence of the CpG islands in c5 and the overlapping cosmid c12 suggested that c5 indeed contained at least one candidate gene for MTS.

5 To search for MTS, the DNA sequences of EcoRI fragments from cosmid c5 were determined. When these sequences were compared against sequences from GenBank, two distinct regions of c5 were identified that were similar to a region of a previously identified gene encoding human cyclin-dependent kinase 4 (Cdk4) inhibitor, or p16 (Serrano et al., 1993). These two genes were candidates for MTS, and were named MTS1 and MTS2. MTS1 was located near the end of
10 cosmid c5 closest to the chromosome 9p telomere, while MTS2 was located near the centromeric end of c5. See Figure 4B. A cosmid map showing the position of MTS1 and MTS2, as well as PIs 1062, 1063 and 1069 is shown in Figure 4A.

A detailed comparison of genomic sequence of MTS1 from c5 with the p16 mRNA sequence revealed that MTS1 contained a stretch of 307 bp that was identical to a portion of the
15 p16 coding sequence. This stretch of nucleotides in MTS1 was flanked by recognizable splice junction sequences. Further characterization of MTS1 showed that it included the entire coding sequence of p16 plus two introns (Figures 5A and 5B and Figures 6A and 6B). Intron 1 was located 126 bp downstream from the translational start site; intron 2 was located 11 bp upstream from the translational stop site. The two introns divided the coding sequence of MTS1 into three
20 regions, a 5' region of 126 bp (coding exon 1), a middle region of 307 bp (coding exon 2), and a 3' region of 11 bp (coding exon 3). SEQ ID NO:3 sets forth nucleotide sequence for the 5' region, exon 1 and part of intron 1 for MTS1. SEQ ID NO:4 sets forth the nucleotide sequence for part of intron 1, exon 2 and part of intron 2 for MTS1.

MTS2 contained a region of DNA sequence nearly identical to p16 that extended from the 5'
25 end of coding exon 2 roughly 211 bp toward intron 2 (Figure 7A). However, the sequence similarity decreased until a point 51 bp upstream of intron 2 in MTS1 which corresponds to the location of the final codon of MTS2 (Figure 8). Comparison of sequences from MTS1 and MTS2 (Figure 8) showed that the sequence similarity between these two genes also extended nearly 40 nucleotides upstream from the 3' splice junction of intron 1. Thus, portions of noncoding DNA
30 were more conserved than some areas of presumptive coding DNA. To exclude the possibility that the sequence divergence in coding DNA might be a cloning artifact, PCR primers were designed to

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amplify specifically across the sequence divergence point of MTS2. These primers amplified a fragment of the predicted size from cosmid, P1 and genomic DNA. Therefore, the divergent sequence located near the 3' end of exon 2 in MTS2 is a *bona fide* genomic sequence. SEQ ID NO:5 sets forth the nucleotide sequence for part of intron 1, "exon 2" and "intron 2" for MTS2.
5 SEQ ID NO:15 sets forth the cDNA sequence for MTS2.

The occurrence of two closely related genes on cosmid c5 suggested that other related genes might exist in this region. To test this possibility, Southern blots were prepared from restriction enzyme digests of cosmids c5, c12, c59, P1s 1063 and 1060, and human genomic DNA. These blots were probed with a fragment containing most of exon 2 from MTS1, including the region
10 shared with MTS2. Two EcoRI fragments were detected with the probe in both cloned DNA and genomic DNA. This result was consistent with the presence of two p16-like genes in the genome, MTS1 and MTS2. It is also consistent with the now known presence of MTS1E1 β which is an alternate form of MTS1 - containing Exons 2 and 3 but not Exon 1 of MTS1.

15

EXAMPLE 7

Isolation and Structure of MTS1E1 β

Isolation of MTS1E1 β

Clones that contained MTS1E1 β were isolated by hybrid selection using the complete MTS1
20 cDNA as a probe and by conventional cDNA library screening. Conventional cDNA library screening was performed using a probe derived from exon 2 of MTS1. One million clones were screened from each of fetal brain, normal breast and lymphocyte-derived libraries. A hybridizing cDNA clone was isolated from the lymphocyte library. The clone was sequenced and shown to contain E1 β . It also contained exon 2 (E2) and exon 3 (E3) of MTS1. Hybrid selection-derived
25 cDNA clones were isolated by incubating cDNA derived from ovarian tissue with cosmid c5. The cosmid was labeled with biotin and made to be single-stranded. Hybrids between c5 and the cDNA were allowed to form and then the biotinylated cosmid was captured using streptavidin-coated magnetic particles. The selected cDNA was eluted from the cosmid, amplified by PCR, cloned and sequenced. The cDNA clones were similar to those isolated by library screening in that
30 they contained E1 β , E2 and E3. None of the clones contained the previously described exon 1 (see SEQ ID NO:3). The sequence for MTS1E1 β cDNA is set forth in SEQ ID NO:13.

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GAC GTG CGC GAT GCC TGG GGC CGT CTG CCC GTG GAC CTG GCT GAG GAG	336
Asp Val Arg Asp Ala Trp Gly Arg Leu Pro Val Asp Leu Ala Glu Glu	
100	105
	110
CTG GGC CAT CGC GAT GTC GCA CGG TAC CTG CGC GCG GCT GCG GGG GGC	384
Leu Gly His Arg Asp Val Ala Arg Tyr Leu Arg Ala Ala Gly Gly	
115	120
	125
ACC AGA GGC AGT AAC CAT GCC CGC ATA GAT GCC GCG GAA GGT CCC TCA	432
Thr Arg Gly Ser Asn His Ala Arg Ile Asp Ala Ala Glu Gly Pro Ser	
130	135
	140
GAC ATC CCC GAT TGA	447
Asp Ile Pro Asp *	
145	

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 149 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Glu Pro Ser Ala Asp Trp Leu Ala Thr Ala Ala Ala Arg Gly Arg	
1	5
	10
	15
Val Glu Glu Val Arg Ala Leu Leu Glu Ala Val Ala Leu Pro Asn Ala	
20	25
	30
Pro Asn Ser Tyr Gly Arg Arg Pro Ile Gln Val Met Met Met Gly Ser	
35	40
	45
Ala Arg Val Ala Glu Leu Leu Leu His Gly Ala Glu Pro Asn Cys	
50	55
	60
Ala Asp Pro Ala Thr Leu Thr Arg Pro Val His Asp Ala Ala Arg Glu	
65	70
	75
	80
Gly Phe Leu Asp Thr Leu Val Val Leu His Arg Ala Gly Ala Arg Leu	
85	90
	95
Asp Val Arg Asp Ala Trp Gly Arg Leu Pro Val Asp Leu Ala Glu Glu	
100	105
	110
Leu Gly His Arg Asp Val Ala Arg Tyr Leu Arg Ala Ala Gly Gly	
115	120
	125
Thr Arg Gly Ser Asn His Ala Arg Ile Asp Ala Ala Glu Gly Pro Ser	
130	135
	140

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Asp Ile Pro Asp *
145

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1149 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
 (A) ORGANISM: Homo sapiens

(ix) FEATURE:
 (A) NAME/KEY: intron
 (B) LOCATION: 1..890

(ix) FEATURE:
 (A) NAME/KEY: exon
 (B) LOCATION: 891..1016

(ix) FEATURE:
 (A) NAME/KEY: intron
 (B) LOCATION: 1017..1149

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TCCCCCGCCC GTWTTAAWTA AACCTCATCT TTCCAGAGTC TGTTCTTATA CCAGGAAATG	60
TACACGTCTG AGAAACCCCTT GCCCCAGACA GTCGTTTTAC ACGCAGGAGG GGAAGGGGAG	120
GGGAAGGAGA GAGCAGTCCT TTTCTCCAAA AGGAATCCTT NGAACTAGGG TTTCTGACTT	180
AGTGAACCCC GCGYTCCTGA AAATCAWGGG TTGAGGGGGT AGGGGGACAC TTYCCTAGTC	240
GYACAGSTKA TTTCGMTYCT CGGTGGGGCT CTCACAMCTA GGAAAGAATW GTTTGCTTT	300
TTCTTATGAT TAAAAGAAGA AGCCATACTT TTCCCTATGA CACCAAACAC CCCGATTCAA	360
TTTGGCAGTT AGGAAGGTTG TATCGCGGAG GAAGGAAACG GGGCGGGGGC GGATTTCTTT	420
TTTAACAGAG TGAACGCACT CAAACACGCC TTTGCTGGCA GGCGGGGGGA GCGCGGCTGG	480
GAGCAGGGGA GGCGGGAGGG CGGTGTGGGG GGCAGGTGGG GAGGAGCCCA GTCCTCCTTC	540
CTTGCCAACG CTGGCTCTGG CGAGGGCTGC TTYCGGCTGG TGCCCCCGGG GGAGACCCAA	600
CCTGGGGCGA CTTCAAGGGGT GCCACATTG CTAAGTGCTC GGAGTTAATA GCACCTCCTC	660